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The Effects of Prey Items Diversity and Digestible Materials in Stomach on Digestive Tract Length in *Hylarana guentheri*

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Abstract Difference in environmental condition shapes variation in digestive tract length in evolutionary process. In particular, environmental difference results in variation in food resource among different habitats, and thereby affecting energy intake and energy allocation. The digestive theory predicts that animals foraging high indigestible materials of stomach contents can promote the increased gut dimensions. Here, we studied variation in digestive tract and gut length across six *Hylarana guentheri* populations at different altitudes and latitudes to test the prediction of the digestive theory. We found that altitude and latitude did not affect variation in relative size of digestive tract and gut among populations. We also found that relative size of digestive tract and gut did not be correlated with diversity of prey items, but negatively correlated with proportion of digestible materials. Our findings suggest that individuals foraging less digestible materials display relatively longer digestive tract than individuals foraging more digestible materials.

Keywords altitude, digestive tract, digestible materials, diversity of prey items, *Hylarana guentheri*

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1. Introduction

The digestive theory predicts that animals foraging high contents of digestible materials lead to decrease gut dimensions (Penry and Jumars, 1987). In particular, animals change their digestive morphology traits to meet food availability variation in order to maximize overall energy return (Sibly, 1981). There are evidences that the consumption of flavorous food can promote larger guts than the consumption of granivorous food in rodents (Hansson, 1985; Hansson and Jaarola, 1989). For species of frogs, individuals foraging indigestible materials possess longer digestive tracts than individuals preying on digestible materials (Nǔnez *et al.*, 1982; Naya *et al.*, 2009; Lou *et al.*, 2013; Ma *et al.*, 2016).

Environmental differences can shape variation in morphology, physiology and behavior in organisms (Nǔnez *et al.*, 1982; Jin *et al.*, 2016; Lüpold *et al.*, 2017; Shultz and Burns, 2017; Alton *et al.*, 2017; Huang *et al.*, 2018; Wang and Liao, 2018; Rodrigues *et al.* 2018; Yu *et al.*, 2018; Samuk *et al.*, 2018; Zhong *et al.*, 2018; Inostroza-Michael *et al.*, 2018; McCullough *et al.*, 2018; Mai and Liao, 2019; Cai *et al.*, 2019; Wang *et al.* 2019; Hussain *et al.* 2019). It has been shown that length of digestive tract varies with altitude across populations in animals (Hammond *et al.*, 1999; Naya *et al.*, 2009; Ma *et al.*, 2016; Wang *et al.*, 2017). For instance, low abundance of prey items and high indigestible materials from of stomach contents from low temperature and short active period at high altitude result in producing longer digestive tract in frogs (Wang *et al.*, 2017). By contrast, an increase in gut length is accompanied by the increased

temperature among *Bufo andrewsi* populations (Ma *et al.*, 2016). Hence, more studies evaluating digestive tract variation associated with diversity of prey items and digestible materials of stomach contents across populations at different altitudes and latitudes in frogs are needed.

The Günther's frog, *Hylarana guentheri* (Anuar: Ranidae) is widely distributed in China and its distribution ranges from 0 to 1100 m a.s.l. Previous studies have investigated advertisement calls (Fei *et al.*, 2010; Li *et al.*, 2010), testis size asymmetry (Liu *et al.*, 2011) and brain size variation among populations (Gu *et al.*, 2017). So far, little information on variation in digestive tract length associated with diversity of prey diets and digestible materials of stomach contents across populations in *H. guentheri* is available. Here, we explored variation in digestive tract length among six populations along a geographical gradient. We also tested the prediction of the digestion theory whether length of digestive tract displays a negative correlation with the proportion of digestible materials, and a positive correlation with diversity of prey items across populations.

2. Materials and Methods

We captured a total of 68 males from six populations in paddy fields, pools and neighboring green sward by hand at night during the breeding season at different sites across Hunan, Hubei and Sichuan provinces in China between June and August in 2016 (Figure 1). We confirmed all individuals to be adults and the sexes were determined by direct observation of secondary sexual characteristics (the black throat that is present only in adult males and on the inflated body, which is characteristic of adult females carrying the eggs) (Liao and Lu, 2010). Each individual immediately was kept at a refrigerator with temperature of -20℃ and taken to the laboratory. Subsequently we sacrificed all individuals using single-pithing and preserved them in 4% buffered formalin in a phosphate buffer (Zhao *et al.*, 2018).

After two months, body size (snout-vent length, SVL) of each individual was measured to the nearest 0.01 mm using a calliper and digestive tract was removed for further measurements. The length of digestive tract (i.e., the beginning of the esophagus to vent length) and gut of the specimen was measured to the nearest 0.01 mm using a vernier calliper for three times. We calculated the final value as mean of these measurements in the analyses. There was a high correlation between repeated measures for length of digestive tracts and guts (both $R > 0.94$). The preservation time do not affect length of digestive tracts (see details in Lou *et al.*, 2013). All dissections and measurements were performed by one person (Yu JP).

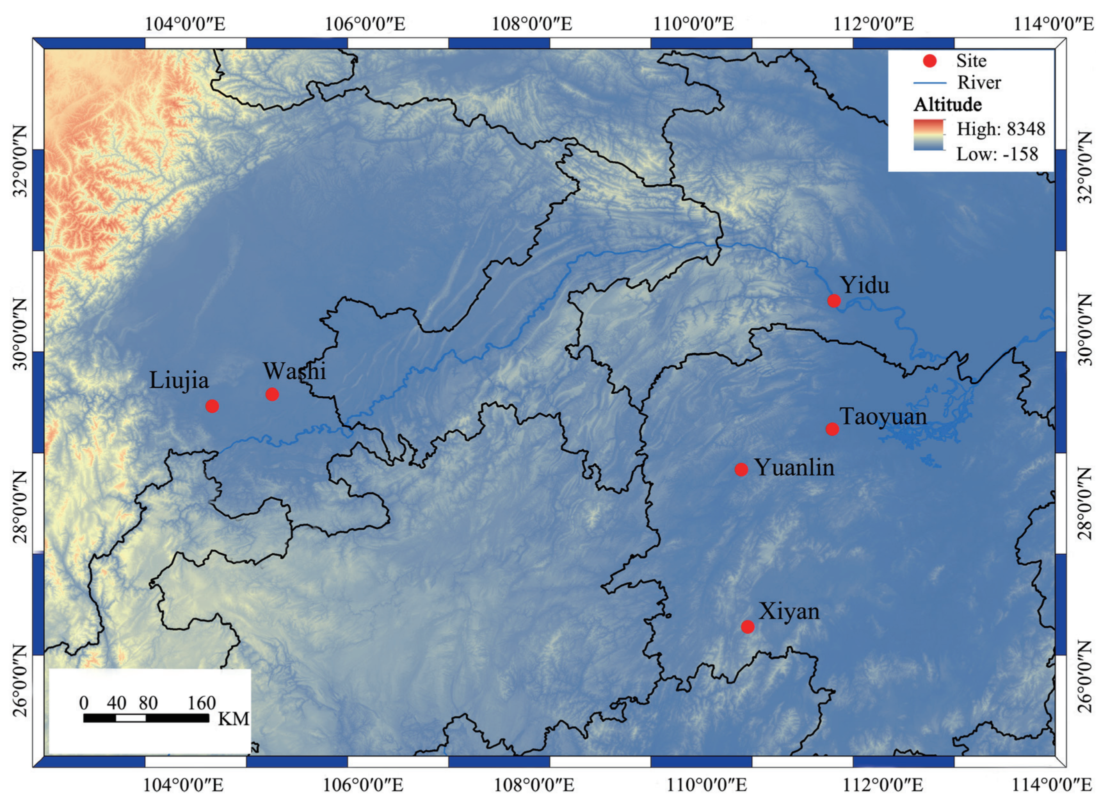

Figure 1 Locations of sampled populations of six *Hylarana guentheri* populations in China.

The stomach contents of each individual were collected in October in 2016. Prey items were subsequently placed in a Petri dish and identified under a stereoscopic microscope, and reference slides were from wings, antenna and legs (Khatiwada *et al.*, 2016). All prey items were then identified to the lowest possible taxonomic level. We considered all prey items (e.g., rice plant weevil, root weevil, rice borer, stem borer, grasshoppers, crickets, insect larvae, leaf hoppers, aphids, and mole crickets, rice thrips, ants, ladybird beetles, and dragonflies) as Order to calculate diversity of prey items and proportion of digestible materials. We first determined number of prey items and presence of particular prey categories in stomach contents within each individual. We then calculated prey composition for each frog individual, expressed as relative frequency (%) (PRF = observed occurrence of item/observed occurrence of all items; Wang *et al.*, 2017). Finally, we calculated average per prey category within each population and used the Shannon-Wiener' diversity index to estimate the diversity of prey items of stomach contents. The diversity of prey items (H) was obtained using an ellipsoid model: $H = -\sum_{i=1}^{s}[(\mathrm{PRF}) \times (\ln \mathrm{PRF})]$. Species richness (s) is based on the number of species present in the stomach (Magguran, 2004). We determined the proportion of digestible materials based on occurrence of spiders, insect larvae and angleworm in stomach contents. The proportion of digestible materials index (D) was used as follows: D = occurrence of spiders, insect larvae and angleworm/ occurrence of all prey items.

All analyses were performed using SPSS 22.0 (Statistical Product and Service Solutions Company, Chicago, USA). Body size, digestive tract and gut length were $\log_{10}$-transformed to improve homogeneity of variances. Relative digestive tract and gut length were estimated from digestive tract and gut length divided by body size. We used one-way ANOVA to test differences in body size, digestive tract and gut length among populations. To test the effects of latitude and altitude on variation in relative size of digestive tract and gut across populations, we performed Linear Mixed Models (LMMs) treating digestive tract or gut length as a dependent variable, population as a random factor, latitude and latitude as fixed factors, and body size as a covariate. We also analyzed the relationships between relative size of digestive tract or gut and both diversity of prey items and proportion of digestible materials among populations using a regression analysis.

3. Results

Body size, digestive tract and gut length significantly differed among six populations (Table 1; body size: $F_{5,62} = 10.059$, $P < 0.001$; digestive tract: $F_{5,62} = 3.870$, $P = 0.004$; gut: $F_{5,62} = 2.258$, $P = 0.06$). The LMMs revealed that altitude and latitude did not affect variation in relative size of digestive tract (altitude: $F_{1,64} = 1.862$, $P = 0.177$; latitude: $F_{1,64} = 0.846$, $P = 0.361$) and relative size of gut (altitude: $F_{1,64} = 0.627$, $P = 0.432$; latitude: $F_{1,64} = 0.980$, $P = 0.326$). The length of digestive tract and gut was positively correlated with body size (Figure 2; digestive tract, $F_{1,64} = 17.846$, $P < 0.001$; gut: $F_{1,64} = 13.234$, $P = 0.001$).

The indices of diversity of prey items and proportion of digestible materials across six populations ranged from 1.67 to 2.15 and 11.76% to 55.56%, respectively. The diversity of prey items did not be correlated with relative size of digestive tract

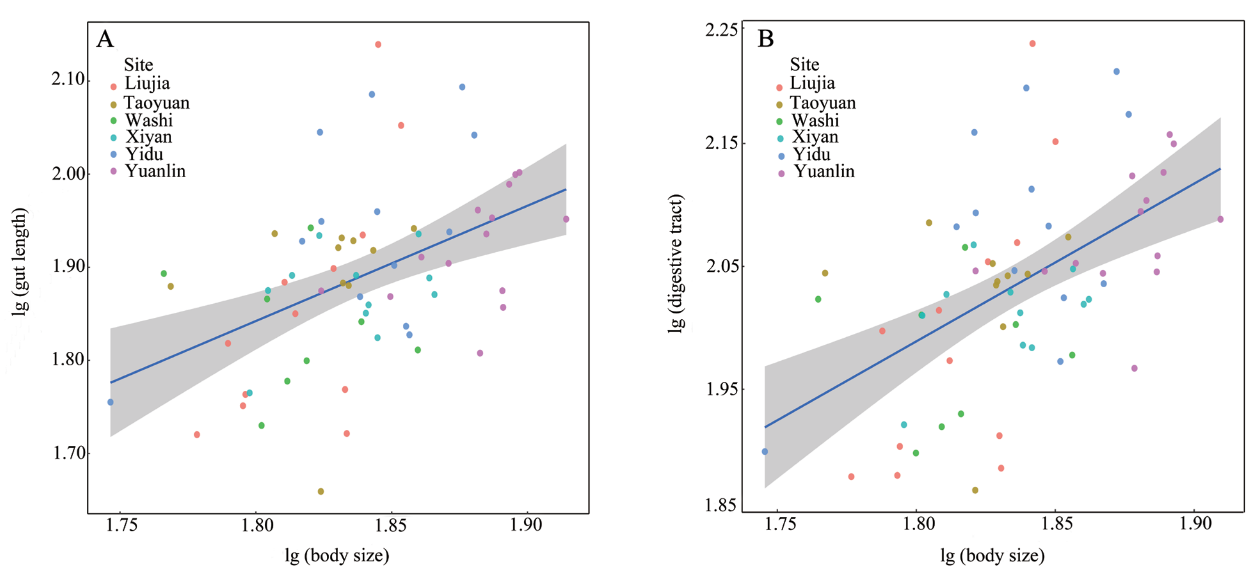

Figure 2 The relationship between length of gut or digestive tract and body size among six *Hylarana guentheri* populations in China.

Table 1 Altitude, latitude, temperature, mean body size, gut, digestive tract length, proportion of digestible materials, and diversity of prey items for six *Hylarana guentheri* populations.

Sites	Altitude	Latitude	Temperature (℃)	Mean body size (mm)	Gut length (mm)	Digestive tract length (mm)	Proportion of digestible materials (%)	Diversity index of prey items
Yidu	84	30.39	17	69.47±5.24 *n* =13	89.6 ±21.39 *n* =13	124.13±24.82 *n* = 13	11.76	1.83
Washi	348	29.31	18	65.45±4.13 *n* = 8	68.75±10.81 *n* = 8	96.13 ±12.87 *n* = 8	51.28	1.69
Liujia	329	29.17	18	65.88±3.65 *n* = 12	75.6 ±26.14 *n* = 12	102.79±29.86 *n* = 12	28.57	1.23
Xiyan	376	26.62	16.6	68.58±3.68 *n* = 11	74.79±8.01 *n* = 11	103.37±8.86 *n* = 11	55.56	1.67
Yuanlin	113	28.44	16.9	75.99±3.87 *n* = 14	84.03±11.28 *n* = 14	121.21±14.25 *n* = 14	18	2.15
Taoyuan	40	28.9	17	67.16±3.6 *n* = 10	78.34±12.34 *n* = 10	107.98±13.36 *n* = 10	17.24	1.68

(Figure 3; relative size of digestive tract = 0.295 diversity of prey items + 1.023, t = 1.490, F = 2.219, P = 0.211) and relative gut size (relative gut size = 1.130 diversity of prey items + 0.620, t = 0.936, F = 0.876, P = 0.402). We also found a negative correlation between proportion of digestible materials and relative size of digestive tract (Figure 4; relative digestive tract size = -0.005 proportion of digestible materials + 1.739, t = -2.898, F = 8.398, P = 0.044), but tended to correlate negatively with relative gut size (relative gut size = -0.003 proportion of digestible materials + 1.247, t = -2.374, F = 5.637, P = 0.076).

4. Discussion

Our study demonstrates that altitude and latitude do not affect variations in relative size of digestive tract and gut among *H. guentheri* populations. Consistent with the prediction of the digestive theory, the relative size of digestive tract is negatively with proportion of digestible materials, suggesting that indigestible food can promote long digestive tract for this frog.

To deal with environmental changes, variation in organ morphology and size in animals are basis of local food resource availability and diversity (Hammond *et al.*, 1999; Liao *et al.*, 2013; Liao *et al.*, 2015; Jiang *et al.*, 2015; Mai *et al.*, 2017; Liu *et al.*, 2018). In particular, abiotic environments associated with altitude (e.g., temperature, water availability) can change biotic conditions (e.g., vegetation cover, prey availability) and affect foraging behavior of individuals (e.g., width and composition of trophic niche), and ultimately changing individuals' digestive features (e.g., gut morphology) (Hammond *et al.*, 1999). For instance, individuals from populations consuming greater plant materials (e.g., folivorous food) exhibit longer guts than individuals from populations predating on granivorous food in the bank voles (*Clethrionomys glareolus*) (Hansson, 1985). For species of anurans (*Bufo spinulosus, P. pleuraden, F. limnocharis*), individuals living in lower altitudes with higher environmental temperature and higher prey availability have shorter guts than those living in higher altitudes with lower environmental temperature and lower prey availability (Naya *et al.*, 2009; Lou *et al.*, 2013; Wang *et al.*, 2017). In this study, we did not find that relative size of digestive tract and gut was affected by altitude and latitude. Actually, all individuals were collected from six populations along a 420-km latitudinal and 341-m altitudinal transect where small geographical ranges made them to experience similar environmental temperature (ranging from 16.9 to 18.0℃) and prey availability, and thus leading to the non-significant effects of altitude and latitude on length of digestive tract and gut. In future, we used more samplings and populations to study digestive tract variation along geographical ranges in this species.

There are evidences that an increase in relative size of digestive tract increases with body size in most species of anurans due to growth (Naya *et al.*, 2009; Lou *et al.*, 2013; Wang *et al.*, 2017). Meanwhile, energy requirements may result in variations in digestive tract per unit body mass among individuals (Pulliainen, 1976). Larger individuals need more energy than smaller individuals, and relatively longer digestive tracts in larger frogs can probably be explained by more energy requirements (Lou *et al.*, 2013). In this study, there was a positive correlation between body size and digestive tract length, suggesting that growth, rather than ecological or evolutionary selection, can explain difference in length of digestive tract..

Previous studies have suggested that less digestible materials and/or diverse diets lead to relatively shorter digestive tract in animals (Nŭnez *et al.*, 1982; Naya *et al.*, 2009; Lou *et al.*, 2013). For

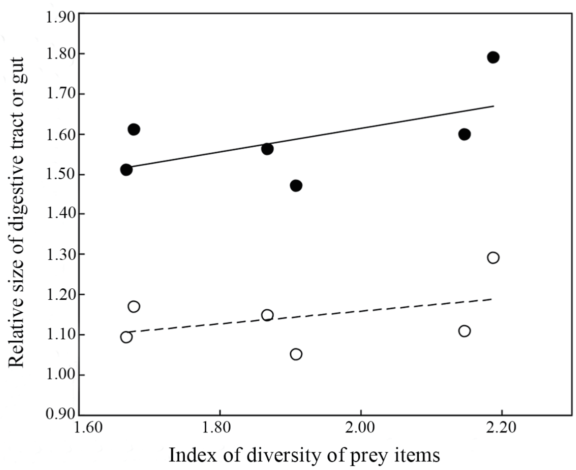

Figure 3 The relationships between diversity of prey items and relative size of digestive tract (full circles and solid line) or gut (empty circles and dotted line) among six *Hylarana guentheri* populations in China. Relative size of digestive tract and gut results from digestive tract and gut length divided by SVL.

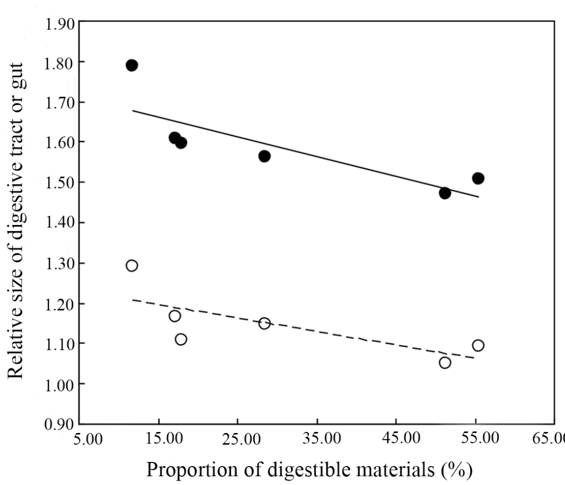

Figure 4 The relationship between proportion of digestible materials and relative size of digestive tract (full circles and solid line) or gut (empty circles and dotted line) among six *Hylarana guentheri* populations in China. Relative size of digestive tract and gut length results from digestive tract and gut length divided by SVL.

instance, individuals increase area and length of digestive tract to digest and absorb more nutrients via forging more diverse diets and indigestible food (Penry and Jumars, 1987). For *F. limnocharis,* individuals foraging more diverse prey diets possess relatively longer digestive tracts than individuals foraging less diverse prey diets (Wang *et al.*, 2017). Here, we did not find a positive correlation between relative size of digestive tract and diversity of prey items, suggesting that diverse prey diets can explain variation in length of digestive tract. Consistent with the prediction of the digestive theory, we found that proportion of digestible food was negatively correlated with digestive tract length among populations, which suggested that individuals forging more digestible materials possessed relatively shorter digestive tract.

In conclusion, because our samplings are collected on the low altitudinal range, and small sample size in each population, high-altitude and/or -latitude individuals do not display relatively longer digestive tract than low-altitude and/or -latitude individuals. Moreover, individuals foraging higher proportion of digestible food display relatively shorter digestive tracts, which support the digestive theory.

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